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The macrophage chemotactic activity of *Edwardsiella tarda* extracellular products

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Abstract

The chemoattractant capabilities of *Edwardsiella tarda* extracellular products (ECP) were investigated from two isolates, the virulent FL6-60 parent and less virulent RET-04 mutant. Chemotaxis and chemokinesis were assayed *in vitro* using blind well chambers with peritoneal macrophages obtained from Nile tilapia, *Oreochromis niloticus*, 5 days following squalene injection. Non-purified ECP derived from both isolates stimulated predominantly chemokinetic migration of macrophages. Additionally, the ECP were semi-purified by high pressure liquid chromatography. The FL6-60 parent ECP yielded higher molecular weight components than did the ECP from the RET-04 mutant. The chemotactic activity of the macrophages for both the FL6-60 parent and RET-04 mutant semi-purified ECP was increased over the non-purified ECP and overall migration was primarily chemotactic. Exposure to ECP derived from virulent and less virulent *E. tarda* isolates promoted chemokinetic movement of macrophages that may be involved in inflammatory responses of Nile tilapia to *E. tarda* infection.

Keywords: chemokinetic, chemotaxis, *Edwardsiella tarda*, extracellular products, macrophage, Nile tilapia.

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Introduction

Edwardsiella septicaemia occurs in numerous freshwater and marine fish species economically important to aquaculture including Japanese eel, *Anguilla japonica* Temminck & Schlegel, red sea bream, *Pagrus major* (Temminck & Schlegel), Japanese flounder, *Paralichthys olivaceus* (Temminck & Schlegel), channel catfish, *Ictalurus punctatus* (Rafinesque), and Nile tilapia, *Oreochromis niloticus* (L.) (Hoshina 1962; Meyer & Bullock 1973; Austin & Austin 1999; Plumb 1999a,b). *Edwardsiella tarda* is recognized as a common inhabitant of aquatic ecosystems and the bacterium has been isolated globally from animal hosts and environmental samples of water and sediments (Plumb & Evans 2006). *Edwardsiella* septicaemia may vary in severity and clinical manifestations in different species, but this bacterium has been implicated in extensive losses of cultured fish throughout the world (Thune, Stanley & Cooper 1993; Plumb & Evans 2006). Despite the potentially serious threat posed by *E. tarda* to fish, the nature of the host–pathogen interaction has yet to be fully elucidated.

In order to develop effective prevention and treatment strategies, an understanding of the factors contributing to the virulence capabilities of a pathogen and the immune responses leading to protection is crucial. Factors such as production of extracellular proteins, cell-associated toxins and avoidance of host cell immune mechanisms have been associated with the pathogenesis of *E. tarda* (Thune *et al.* 1993; Plumb & Evans 2006). Fish possess numerous humoral- and cell-mediated

innate and acquired mechanisms to defend against bacterial infection (Ellis 1999; Magnadóttir 2006).

Edwardsiellosis in fish is characterized by inflammation of several organs including the head kidney, liver and spleen (Miyazaki & Egusa 1976a,b; Miyazaki & Kaige 1985). Macrophages are crucial to inflammatory reactions and other immune responses through their ability to function in effector, helper and suppressor capacities (Nash, Fletcher & Thomson 1986; Zelikoff, Enanf, Bowser, Squibb & Frenkel 1991). The migration of macrophages from the blood and adjacent tissues to the site of microbial infiltration or injury is a critical initial step in the inflammatory process (Griffin 1984; Weeks, Sommer & Dalton 1988; Weeks-Perkins & Ellis 1995; Klesius & Sealey 1996; Ellis 2001). The movement of macrophages and other leucocytes may involve chemotactic and chemokinetic mechanisms. Chemotaxis refers to the directed migration of cells induced by chemoattractant molecules of either microbial or host origin, while chemokinesis refers to an increase in non-directional, random movement of cells (Obenauf & Smith 1985; Nash *et al.* 1986; Weeks *et al.* 1988; Weeks-Perkins & Ellis 1995; Mañes, Gómez-Moutón, Lacalle, Jiménez-Baranda, Mira & Martínez-A 2005).

Numerous studies detailing the *in vitro* chemotactic response of fish leucocytes to microbially derived chemoattractant substances have been reported (Griffin 1984; Obenauf & Smith 1985; Sharp, Pike & Secombes 1991; Zelikoff *et al.* 1991). Klesius & Sealey (1996) investigated the potential chemotactic and chemokinetic activity of channel catfish macrophages exposed to extracellular products (ECP) from *Edwardsiella ictaluri*. Assays conducted both *in vivo* and *in vitro* indicated that ECP are capable of attracting channel catfish macrophages and these interactions could enhance the understanding of *E. ictaluri* pathogenesis (Klesius & Sealey 1996). Recently, Klesius, Evans & Shoemaker (2007) described the chemoattractant properties of ECP obtained from the fish pathogens *Streptococcus agalactiae* and *Streptococcus iniae* on peritoneal macrophages of Nile tilapia. This research found *S. agalactiae* and *S. iniae* ECP preparations displayed chemotactic and chemokinetic activity, although *S. agalactiae* showed significantly greater ability to induce both chemotaxis and chemokinesis in macrophages (Klesius *et al.* 2007). Although production and excretion of ECP have been implicated in the virulence of *E. tarda* (Ullah & Arai 1983a,b; Suprapto, Nakai & Muroga 1995; Suprapto, Hara, Nakai & Muroga 1996; Tan, Lin, Wang, Joshi, Hew & Leung 2002), information regarding the interactions between these ECP and fish macrophages is lacking. Because *E. tarda* is a major pathogen of tilapia, and tilapia are among the most widely cultured species of food fish, the aim of the present study was to investigate the possible ability of non-purified and semi-purified ECP prepared from a virulent parent and less virulent mutant of *E. tarda* to influence chemotactic and chemokinetic migration of Nile tilapia macrophages *in vitro*.

Materials and methods

Fish

Nile tilapia, *Oreochromis niloticus* (60 ± 5 g, mean weight ± SD), were produced and maintained at the USDA-ARS Aquatic Animal Health Research Laboratory (AAHRL), Auburn, AL, USA. The fish were fed a commercial diet (Aquamax Grower 400, Brentwood, MO, USA)[1] daily to satiation. Two weeks prior to the experiments, the fish were acclimatized in 57-L glass aquaria. The aquaria were provided with flow-through dechlorinated tap water at 26 ± 1 °C at a rate of 0.5 L min^{-1} and aeration with air stones. For the duration of the study, the dissolved oxygen, temperature, pH, salinity, hardness, ammonia and nitrite levels were monitored and maintained within acceptable ranges. During the experiment, the mean ± SD of dissolved oxygen was 5.8 ± 0.5 mg L^{-1}, temperature was 26.2 ± 0.8 °C, pH was 7.1 ± 0.3, salinity was 0.1 ± 0.0% and hardness was 110 ± 10 mg L^{-1} ($CaCO_3$). Ammonia and nitrite concentrations were consistently below the detection limit (0 mg mL^{-1}) during the experiment. The fish were exposed to light and dark intervals of 12:12 h. Feeding was discontinued 1 day preceding collection of squalene-induced peritoneal macrophages.

Preparation of *E. tarda* non-purified ECP

The preparation of *E. tarda* (ECP) was guided by the procedures presented by Klesius, Shoemaker & Evans (1999) describing the formulation of a killed

[1]Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the United States Department of Agriculture.

S. iniae vaccine. The resulting ECP products from *E. tarda* were used to characterize the biological properties from a virulent (FL6-60) parent and less virulent (RET-40) mutant isolate. The virulent FL6-60 parent isolate was obtained from a morbid striped bass, *Morone saxatilis* (Walbaum) (Baya, Romalde, Green, Navarro, Evans, May & Toranzo 1997). This isolate was received courtesy of Dr A.M. Baya (Animal Health Diagnostic Laboratory, Maryland Department of Agriculture, College Park, MD, USA). RET-04 is a mutant of FL6-60 developed through a rifampicin-resistance strategy as described by Schurig, Roop, Bagchi, Boyle, Buhrman & Sriranganathan (1991), Klesius & Shoemaker (1999) and Evans, Klesius & Shoemaker (2006). The FL6-60 parent isolate was determined to be more virulent than the RET-04 mutant isolate through previous studies (Wiedenmayer, Evans & Klesius 2006). Briefly, 1-mL aliquots of the FL6-60 parent and RET-04 mutant were inoculated, separately, into 5 L of sterile tryptic soy broth (TSB; Difco Laboratories, Sparks, MD, USA) and incubated at 27 °C for 72 h with shaking. The plate counts of the FL6-60 and RET-04 preparations after 72 h incubation were 3.02×10^9 and 1.68×10^9 colony forming units mL^{-1} (CFU mL^{-1}), respectively. The cultures were treated with 10% neutral-buffered formalin (NBF) to yield a final concentration of 3% and allowed to stand at 27 °C for 24 h. After 24 h, the culture was streaked onto sheep blood agar (SBA; Remel, Inc., Lenexa, KS, USA) and then incubated at 27 °C for 72 h to ensure the preparation had been killed. The NBF treated cultures were centrifuged at 7000 ***g*** for 30 min and the cell pellet and culture fluid separated. The culture fluid, containing ECP, was concentrated to 1 L on a 3-kDa Amicon column (S3Y3) using a Millipore Proflux M12 (Millipore, Billerica, MA, USA) and filter sterilized with a 0.22-μm 1-L microbiological filter (Corning, Corning, NY, USA). The protein concentration of the non-purified ECP was determined by the bicinchoninic acid (BCA) method and estimated to be approximately 7.74 ± 1.30 and 6.64 ± 1.66 mg mL^{-1} for the FL6-60 and RET-04 preparations, respectively (BCA Protein Assay Kit; Pierce, Rockford, IL, USA). A *Limulus* amoebocyte lysate (LAL) semi-quantitative assay (Cambrex Bio Science, Inc., Walkersville, MD, USA) was used to determine the endotoxin units (EU) mL^{-1} of lipopolysaccharide (LPS) in the non-purified ECP and the semi-purified ECP from both the parent and mutant *E. tarda* isolates. 0.2 mL of the non-purified and semi-purified ECP was assayed at four different concentrations twice for EU mL^{-1} of LPS. The mean endotoxin concentration was 0.171 and 0.416 EU mL^{-1}, respectively, for non-purified ECP from mutant and parent ECP preparations. Ten EU mL^{-1} is approximately equal to 1 ng mL^{-1}. The approximate amount of LPS in the ECP was 0.0171 and 0.0416 ng mL^{-1}, respectively. The semi-purified ECP from both the mutant and parent had no detectable LPS by the LAL assay. The whole cell fraction and concentrated culture fluid were stored separately at −80 °C until needed in the study.

Semi-purification of ECP

For additional characterization, non-purified ECP were semi-purified via a high-pressure chromatography system (Waters, Milford, MA, USA) outfitted with a Shodex® Protein KW-804 column (Showa Denko K.K., Tokyo, Japan). The total volume of the column (V_t) was 2400 mL while the void volume (V_o) was determined to be 4.5 mL. TSB was analysed as a control. Elution was performed in phosphate-buffered saline (PBS) at a flow rate of 0.5 mL min^{-1}. The elution profile was detected at a wavelength of 280 nm (Waters 996 Photodiode Array Detection, Milford, MA, USA). Following the proper elution period, the semi-purified ECP were collected and pooled by isolate. The molecular weights of the semi-purified ECP were estimated by comparison to molecular weight standards ranging from 1.35 to 670 kDa (Bio-Rad 151–1091, Hercules, CA, USA). The determination of protein concentrations of the semi-purified ECP was attempted by the BCA method; however, the protein amounts fell below the range of detection for the assay. The semi-purified ECP were stored at −70 °C until used.

Macrophage collection

Macrophage enriched exudate was collected from Nile tilapia using the method of Klesius & Sealey (1996). Five Nile tilapia (60 ± 5 g, mean weight ± SD) were intraperitoneally (IP) injected with 250 μL of squalene (Sigma Chemical Co., St Louis, MO, USA). The fish were maintained at the previously described conditions for 5 days, with feeding discontinued 1 day prior to the harvest of the peritoneal exudate. After 5 days, the fish were

killed by immersion in a 300 mg mL^{-1} buffered tricaine methanesulphonate (MS-222; Finquel, Argent Chemical Laboratories, Redmond, CA, USA) solution. The peritoneal cavity of each fish was washed three times with 15 mL of cold sterile PBS using a 20-gauge needle connected to a three-way valve. The collected peritoneal exudates were pooled and centrifuged at 300 ***g*** for 10 min at 4 °C. The supernatant was removed and discarded and the reserved cells suspended in calcium- and magnesium-free Hank's balanced salt solution without phenol red (HBSS; Gibco, Grand Island, NY, USA). The cells were washed once in HBSS as described above and the resulting cell pellet resuspended in 2 mL HBSS. The cell solution was diluted with 9 mL of sterile deionized water to lyse the red blood cells for 20 s, after which 1 mL of 10× HBSS was added. The cells were washed twice in the previously described HBSS at 300 ***g*** for 10 min at 4 °C. The collected cells were enumerated and assessed for viability using a haemocytometer and the trypan blue exclusion assay.

Chemotaxis and chemokinesis assays of non-purified ECP

Chemotaxis assays were performed using the lower surface method detailed by Boyden (1962), as adapted by Klesius & Sealey (1996) and Klesius *et al.* (2007). Nile tilapia macrophage migration in the presence of *E. tarda* ECP was assayed in duplicate with blind well chemotactic chambers (Corning CoStar, Cambridge, MA, USA) and 8-μm pore diameter polycarbonate membrane filters (Nucleopore, Pleasanton, CA, USA). The filters were treated prior to macrophage analysis with RPMI-1640 (Gibco BRL, Grand Island, NY, USA) containing 1% horse serum. To evaluate directional movement of macrophages, the lower chamber compartment was filled with 200 μL of various concentrations of sterile ECP or RPMI-1640 with 1% horse serum as a control. The ECP concentrations contained in the lower compartment were 0%, 10%, 30%, 50% or 70%. The upper chamber compartment was filled with 200 μL of exudate cells with a concentration of approximately 1.0×10^5 cells mL^{-1}. In addition, the checkerboard assay of Zigmond & Hirsch (1973), also employed by Klesius & Sealey (1996), was used to determine chemokinetic and chemotactic activity of Nile tilapia macrophages in the presence of *E. tarda* ECP. For this analysis, ECP were placed in the upper chamber compartment with exudate cells, as well as various concentrations of ECP in the upper, lower, or both compartments for a volume of 200 μL in each compartment. The ECP concentrations placed in the upper, lower, or both chamber compartments were 0%, 10%, 30%, 50% or 70%. For both the lower surface and checkerboard procedures, the chambers were incubated at 25 °C on a horizontal platform shaker at 100 rpm. After 90-min incubation, the filters were removed from the chamber, inverted and placed on a precleaned slide. The filter was attached to the slide using clear fingernail polish and the slide stained with Protocol Hema 3 stain (Fisher Scientific Co., LLC, Kalamazoo, MI, USA), in a manner similar to Weeks *et al.* (1988) and Klesius & Sealey (1996). Enumeration of migrating macrophages was achieved by counting five fields of view on the bottom surface of the filters with a light microscope at 400× magnification. The mean number of macrophages per field of view and SE were calculated.

Chemotaxis and chemokinesis assays of semi-purified ECP

Chemotaxis and chemokinesis assays were performed utilizing semi-purified ECP obtained from both the FL6-60 parent and RET-04 mutant *E. tarda*. The procedures detailing the lower surface and checkerboard assays were executed as described previously for analysis utilizing non-purified ECP, with the exception of the ECP concentrations. For this study, the ECP concentrations placed in the upper, lower, or both chamber compartments were 0%, 50% or 70%.

Statistical analysis

Chemotactic and chemokinetic data were analysed using SAS software (SAS Institute, Cary, NC, USA) by one-way analysis of variance (ANOVA) with Duncan's multiple range test for significance and by *t*-test. Significant differences were determined at $P < 0.05$.

Results

Determination of chemotactic activity for *E. tarda* FL6-60 parent and RET-04 mutant non-purified ECP

The FL6-60 parent ECP induced greater migration of macrophages as the concentration of ECP in the

lower chamber was increased. The ECP concentrations of 30%, 50% and 70% in the lower chamber produced mean macrophage counts differing significantly ($P < 0.0001$) from those obtained from ECP concentrations of 0% and 10% in the lower chamber (Table 1). The RET-04 mutant ECP also induced greater migration of macrophages as the concentration of ECP in the lower chamber was increased. The ECP concentrations of 10%, 30%, 50% and 70% produced mean macrophage counts differing significantly ($P < 0.0001$) from those obtained from an ECP concentration of 0% in the lower chamber (Table 1). No significant differences were observed for mean macrophage counts between the FL6-60 parent and RET-04 mutant ECP in the lower surface assay (Table 1).

Determination of chemokinetic activity for *E. tarda* FL6-60 parent and RET-04 mutant non-purified ECP

The checkerboard assay was employed to distinguish chemokinetic from chemotactic activities of the non-purified ECP preparations of the FL6-60 parent and RET-04 mutant. With analysis of both ECP, macrophage migration seemed to be primarily chemokinetic in nature. Increases of ECP concentration in the lower chamber did appear to stimulate movement of macrophages across the membrane, but movement of macrophages was also greater when ECP concentrations were higher in the upper chamber (Table 1). While concentrations of 10%, 30% and 50% of both ECP and 70% of the RET-04 mutant ECP in both the lower and upper chambers did not elicit significantly different mean macrophage movements, these means were significantly ($P < 0.0001$) different than those of the 0% ECP concentrations of the control suggesting a lack of neutralization (Table 1). Additionally, mean migration of macrophages observed where the FL6-60 parent ECP concentration was 70% in both the lower and upper chambers was similar to movement seen in chambers where ECP were only contained in the lower chamber (Table 1). Significant differences were observed for mean macrophage counts between the FL6-60 and RET-04 treatments with 30 ($P < 0.0168$) and 50% ($P < 0.0062$) ECP in the upper chamber with 0% ECP in the lower chamber, and with 70% ECP in the upper chamber with 10% ($P < 0.0029$) and 70% ($P < 0.0017$) ECP in the lower chamber (Table 1).

Comparison of semi-purified *E. tarda* FL6-60 parent and RET-04 mutant *E. tarda* ECP molecular weight

The molecular weights of the semi-purified ECP of the FL6-60 parent and RET-04 mutant preparations were determined based on the elution times of five molecular weight standards. The elution times were recorded as 18.629, 23.367, 25.466, 27.199

Table 1 Effect of varying the concentration of non-purified extracellular products (ECP) obtained from the FL6-60 parent and RET-04 mutant isolates of *Edwardsiella tarda* in the upper and lower chemotactic assay chambers on number of migration of Nile tilapia macrophages to the lower chamber

Per cent concentration of ECP in the lower chamber	Per cent concentration of *E. tarda* ECP in the upper chamber				
	0	10	30	50	70
0	6.9 ± 0.6^{i}	$15.6 \pm 2.1^{f,g,h}$	**$9.3 \pm 1.5^{h,i}$**	**$23.2 \pm 2.9^{b,c,d,e,f}$**	$22.4 \pm 2.2^{b,c,d,e,f}$
10	$8.8 \pm 2.2^{h,i}$	$17.8 \pm 1.2^{f,g}$	$22.5 \pm 2.6^{b,c,d,e,f}$	$18.6 \pm 1.6^{f,g}$	**$29.4 \pm 0.8^{a,b,c}$**
30	$23.0 \pm 1.5^{b,c,d,e,f}$	$23.2 \pm 2.1^{b,c,d,e,f}$	$16.4 \pm 1.2^{f,g}$	$26.8 \pm 2.4^{a,b,c,d,e}$	$30.0 \pm 1.9^{a,b}$
50	$29.8 \pm 3.5^{a,b}$	$22.0 \pm 3.3^{c,d,e,f}$	$13.4 \pm 0.7^{g,h,i}$	$19.2 \pm 1.0^{e,f,g}$	$23.3 \pm 2.9^{b,c,d,e,f}$
70	33.4 ± 2.3^{a}	$27.6 \pm 3.3^{a,b,c,d}$	$22.4 \pm 3.0^{b,c,d,e,f}$	$20.6 \pm 4.4^{d,e,f,g}$	**$30.0 \pm 2.8^{a,b}$**
	Per cent concentration of *E. tarda* RET-04 ECP in the upper chamber				
	0	10	30	50	70
0	6.9 ± 0.6^{h}	$11.2 \pm 2.4^{g,h}$	**$18.0 \pm 2.1^{e,f,g}$**	**7.3 ± 2.9^{h}**	$20.4 \pm 2.7^{c,d,e,f}$
10	$15.3 \pm 2.2^{f,g}$	$15.0 \pm 1.9^{f,g}$	$17.4 \pm 1.6^{e,f,g}$	$19.0 \pm 1.7^{d,e,f,g}$	**$16.0 \pm 3.1^{e,f,g}$**
30	$21.0 \pm 3.5^{c,d,e,f}$	$17.8 \pm 3.2^{e,f,g}$	$15.0 \pm 1.7^{f,g}$	$30.2 \pm 1.0^{a,b}$	$27.0 \pm 1.8^{b,c}$
50	$25.6 \pm 1.2^{b,c,d}$	$27.0 \pm 2.9^{b,c}$	$17.5 \pm 4.5^{e,f,g}$	$20.2 \pm 4.0^{c,d,e,f}$	$23.6 \pm 1.8^{b,c,d,e}$
70	36.2 ± 1.4^{a}	$29.8 \pm 3.3^{a,b}$	$17.8 \pm 2.0^{e,f,g}$	$22.8 \pm 0.5^{b,c,d,e,f}$	**$16.2 \pm 0.1^{e,f,g}$**

Mean ± SE of numbers of macrophages in duplicate filters, each read in five fields of view with a light microscope at 400×. Macrophages added to the upper chamber and migration to ECP in the lower chamber were measured. Macrophage values without a letter in common are significantly different ($P < 0.05$). Macrophage values in bold are significantly different ($P < 0.05$) from values observed for FL6-60 parent or RET-04 mutant ECP at the same concentrations.

and 30.241 min for the 670, 158, 44, 17 and 1.35 kDa standards, respectively. The elution profile of the FL6-60 parent ECP preparation revealed one major peak at 26.278 min and two minor peaks at 31.952 and 36.099 min (Fig. 1). The molecular weights were estimated to be approximately 31.62 kDa for the major peak and 3.19 and 0.60 kDa for the minor peaks, respectively, though the third peak was outside the range of the standards. The elution profile of the RET-04 mutant preparation revealed one major peak at 30.537 min and two minor peaks at 36.278 and 40.56 min (Fig. 2). The molecular weights were estimated to be approximately 5.65 kDa for the major peak and 0.55 and 0.10 kDa for the minor peaks, respectively, although the second and third peaks were outside the range of the standards. For both ECP preparations, all peaks were collected at the appropriate elution periods and pooled by isolate for the subsequent migration assays. As a control, TSB was also analysed by HPLC. The elution profile showed two major peaks at 26.517 and 31.055 min and one minor peak at 35.366 min (Fig. 3). The molecular weights were estimated to be approximately 28.70, 4.58 and 0.80 kDa, respectively, but again the third peak was outside the range of the standards.

Determination of chemotactic activity for *E. tarda* FL6-60 parent and RET-04 mutant semi-purified ECP

The lower surface assay was utilized to assess the chemoattractant capabilities of semi-purified ECP of the FL6-60 parent and RET-04 mutant. The semi-purified products were prepared, as previously described, by HPLC and pooled by isolate. The FL6-60 parent ECP elicited significantly ($P < 0.0001$) greater migration of macrophages as the concentration of ECP in the lower chamber was increased from the 0% control to 50% and 70%. Additionally, 70% ECP in the lower chamber

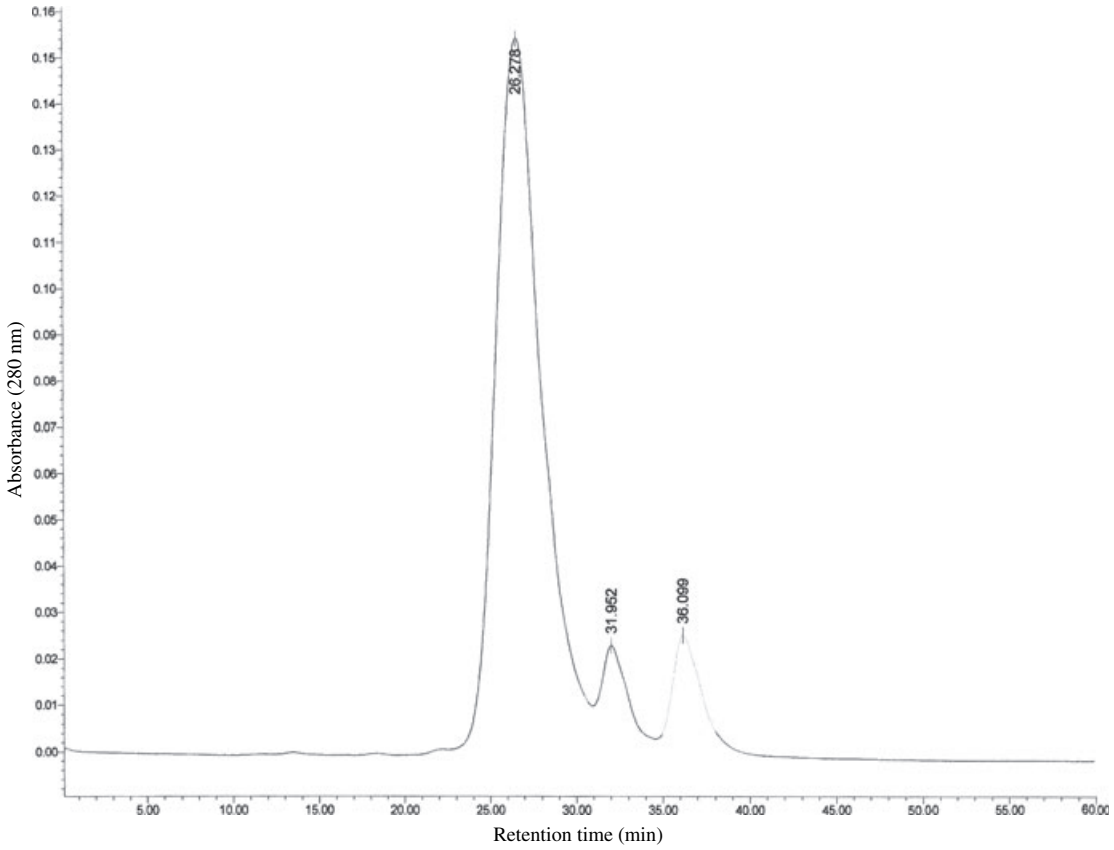

Figure 1 Retention profile (detection at 280 nm) of *Edwardsiella tarda* FL6-60 parent extracellular products from high pressure liquid chromatography in 0.1 M phosphate-buffered saline.

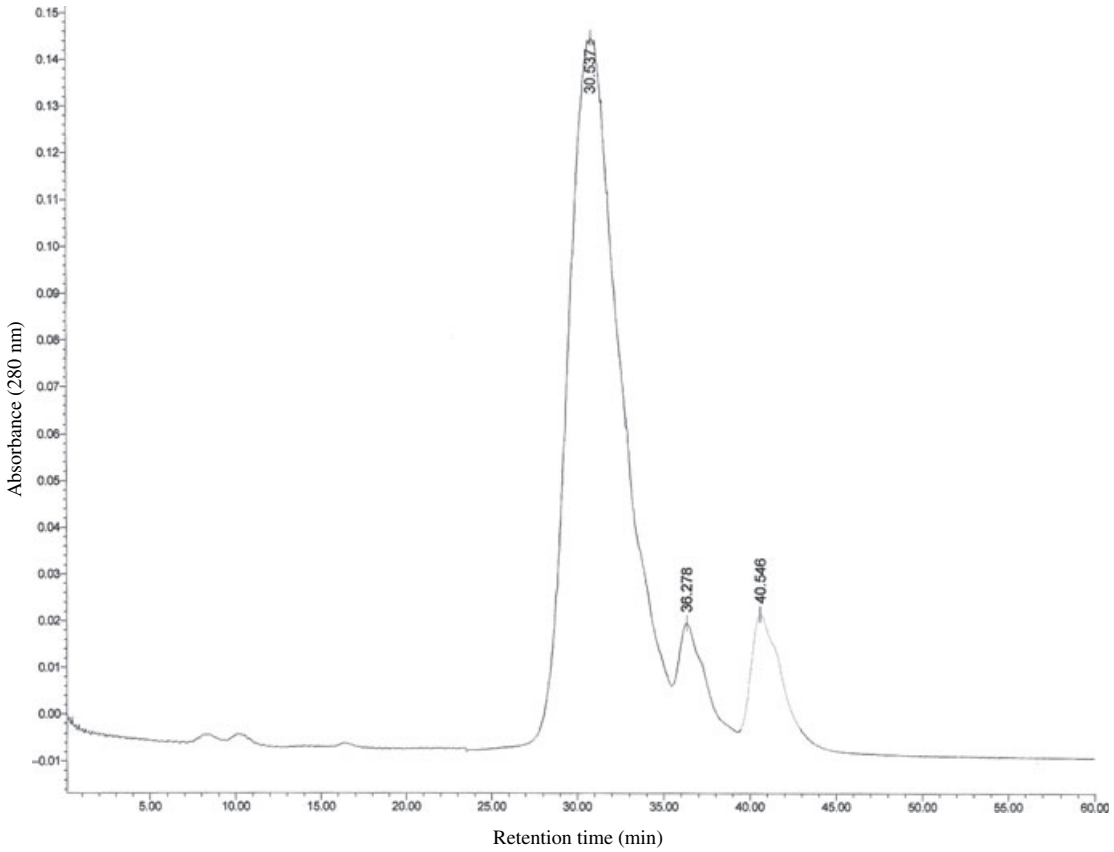

Figure 2 Retention profile (detection at 280 nm) of *Edwardsiella tarda* RET-04 mutant extracellular products from high pressure liquid chromatography in 0.1 M phosphate-buffered saline.

produced significantly ($P < 0.0001$) more macrophage movement than did 50% ECP (Table 2). Similarly, the RET-04 mutant ECP also elicited significantly ($P < 0.0001$) greater migration of macrophages as the concentration of ECP in the lower chamber was increased from the 0% control to 50% and 70%. As with the FL6-60 parent ECP, 70% ECP in the lower chamber produced significantly ($P < 0.0001$) more macrophage movement than did 50% ECP (Table 2). No significant differences were observed for mean macrophage counts between the FL6-60 parent and RET-04 mutant for the lower surface assay (Table 2).

Determination of chemokinetic activity for *E. tarda* FL6-60 parent and RET-04 mutant semi-purified ECP

The semi-purified FL6-60 parent and RET-04 mutant ECP were incorporated in a checkerboard assay to distinguish chemokinetic from chemotactic activities. Both the FL6-60 parent and RET-04 mutant ECP appeared to exhibit chemotactic and chemokinetic activities. In the checkerboard analysis, both ECP preparations showed neutralization of macrophage migration when the ECP concentrations were equal in the lower and upper chambers and mean macrophage counts were not significantly different from those of the 0% ECP concentration control (Table 2). Additionally, increased macrophage migration was observed for both ECP when concentrations were 50% or 70% in the upper chamber and 0% ECP in the lower chamber (Table 2). Significant differences did not occur in the comparison of both ECP in replicates with 50% or 70% concentrations in either the upper or lower chambers to the control, with the exception of significantly ($P < 0.0001$) greater macrophage migration observed with 70% RET-04 mutant ECP in the upper and 50% in the lower chamber over the control (Table 2). Significant differences

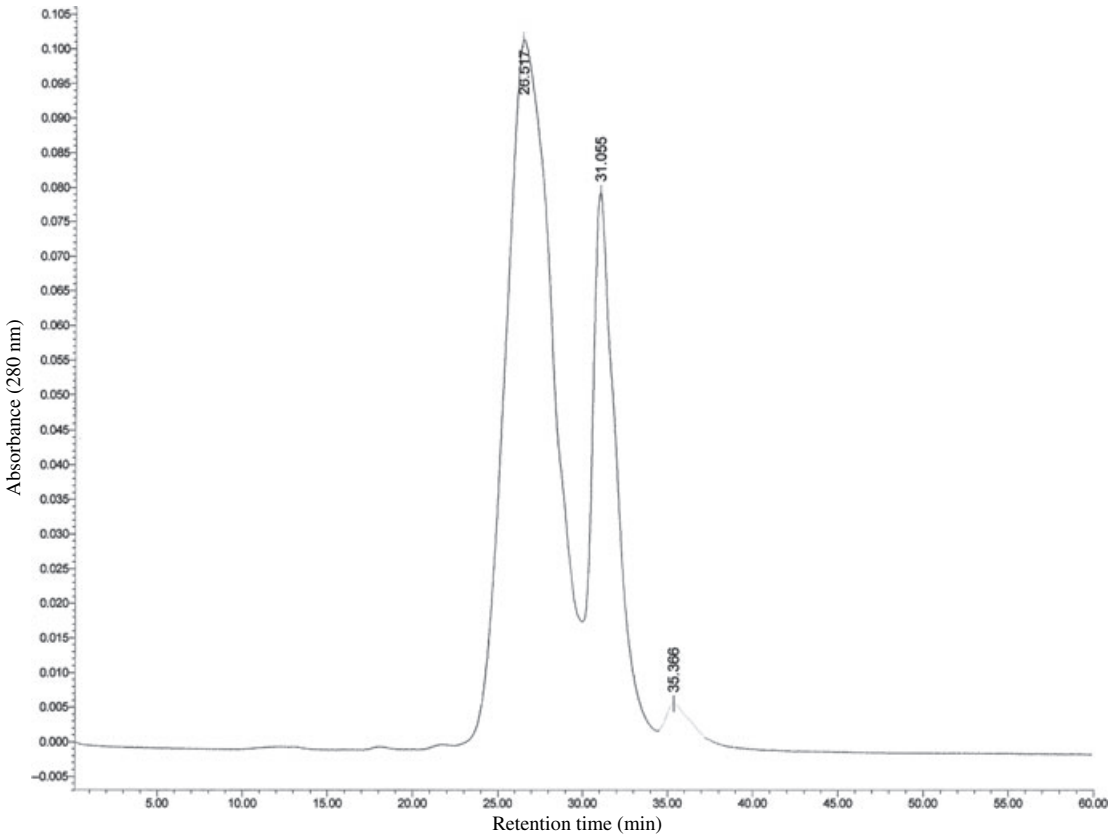

Figure 3 Retention profile (detection at 280 nm) of tryptic soy broth from high pressure liquid chromatography in 0.1 M phosphate-buffered saline.

Table 2 Effect of varying the concentration of semi-purified extracellular products (ECP) obtained from the FL6-60 parent and RET-04 mutant isolates of *Edwardsiella tarda* in the upper and lower assay chemotactic chambers on the number of Nile tilapia macrophages migrating to the lower chamber

Per cent concentration of ECP in the lower chamber	Per cent concentration of *E. tarda* FL6-60 ECP in the upper chamber		
	0	50	70
0	20.6 ± 1.6^{c}	**38.9 ± 2.6^{b}**	**50.7 ± 1.5^{a}**
50	37.8 ± 2.2^{b}	22.2 ± 2.6^{c}	26.3 ± 3.0^{c}
70	47.4 ± 2.3^{a}	26.4 ± 3.0^{c}	20.5 ± 1.6^{c}
	Per cent concentration of *E. tarda* RET-04 ECP in the upper chamber		
	0	50	70
0	20.6 ± 1.6^{e}	**25.4 ± 2.7^{d,e}**	**30.7 ± 4.2^{c,d}**
50	37.4 ± 1.6^{b}	19.8 ± 1.2^{e}	32.3 ± 1.6^{b,c}
70	43.8 ± 2.7^{a}	22.4 ± 4.5^{e}	23.4 ± 1.0^{e}

Mean ± SE of numbers of macrophages in duplicate filters, each read in five fields of view with a light microscope at 400×. Macrophages added to the upper chamber and migration to ECP in lower chamber was measured. Macrophage values without a letter in common are significantly different ($P < 0.05$). Macrophage values in bold are significantly different ($P < 0.05$) from values observed for FL6-60 parent or RET-04 mutant ECP at the same concentrations.

were seen between the FL6-60 and RET-04 treatments with 50% ($P < 0.0029$) or 70% ($P < 0.0083$) ECP concentrations in the upper chamber and 0% ECP concentration in the lower chamber, although the migration behaviour observed for both the FL6-60 parent and RET-04

mutant appeared consistent with chemokinesis for these replicates (Table 2).

Discussion

This study evaluated the ability of ECP derived from a virulent parent and less virulent mutant isolate of *E. tarda* to induce chemotactic and chemokinetic movement of Nile tilapia macrophages. Both non-purified and semi-purified ECP fractions were analysed by varying concentrations of the ECP in blind well chemotactic chambers and assessing macrophage migration. In the lower surface assay of non-purified ECP, results indicated both the FL6-60 parent and RET-04 mutant ECP enhanced movement of macrophages towards increased concentrations of ECP. Weeks *et al.* (1988) reported a potential correlation between chemotactic activity and virulence in studies of *Legionella pneumophila*, suggesting lowered chemoattractant capability of a pathogen was related to increased virulence. In the present study, both the FL6-60 parent and RET-04 mutant ECP did appear capable of stimulating chemotaxis; however, no significant differences in level of migration were observed between the virulent and less virulent fractions. To determine possible chemokinesis, non-purified FL6-60 parent and RET-04 mutant ECP were applied to the chambers in a checkerboard pattern of varying concentrations in both the upper and lower wells. For both ECP, migration appeared to be largely chemokinetic. As the quantity of ECP in the upper compartment was increased, macrophage migration was enhanced, and when ECP concentrations were equal in the upper and lower chambers movement of macrophages was not neutralized. Additionally, as observed in the lower surface assay of the non-purified ECP, few differences between the chemokinetic activity of the virulent FL6-60 parent and less virulent RET-04 mutant ECP were revealed.

Differences existed between the elution profiles of the *E. tarda* FL6-60 parent and RET-04 mutant ECP as generated by HPLC analysis. Three peaks were eluted from the non-purified FL6-60 parent ECP with molecular weights estimated to be 31.62, 3.19 and 0.60 kDa, while the three peaks eluted from the non-purified RET-04 mutant ECP were considerably smaller at estimated molecular weights of 5.65, 0.55 and 0.10 kDa. Because of the small size of most of the respective ECP components, it is possible the molecular weights estimated for these components may not be accurate as the elution times and estimated molecular weights fell outside the range established by the standards. Additionally, the estimated molecular weights of the 0.60, 0.55 and 0.10 kDa components are smaller than the molecular weight cutoff of the column used to concentrate the ECP. The 31.62 kDa component detected in the elution profile of the FL6-60 parent ECP was not observed for the RET-04 mutant ECP, but was comparable in elution time and molecular weight to the first peak identified for the TSB control. This fraction probably did not contribute greatly to the chemoattractant capabilities of the semi-purified FL6-60 parent ECP given the generally similar macrophage migration elicited by both ECP, despite the lack of this component in the RET-04 mutant ECP profile. These results suggest the chemoattractant properties of the FL6-60 parent and RET-04 mutant ECP may be attributed to the small molecular weight components revealed in the semi-purification of both ECP preparations. Notwithstanding the apparent distinction in molecular weight of the semi-purified ECP, few differences between the semi-purified FL6-60 parent and RET-04 mutant ECP were exhibited in the stimulation of macrophage migration as evaluated by the lower surface and checkerboard assays. Similar chemoattractant activity was observed for both semi-purified ECP for all treatments, with the exception of the 50% and 70% ECP concentration in the upper chamber over 0% ECP concentration in the lower chamber replicates where significantly higher migration was seen with the FL6-60 semi-purified ECP over the RET-04 semi-purified ECP at the same concentrations. While similar quantities of protein were detected in the non-purified FL6-60 parent and RET-04 mutant ECP, BCA analysis of the semi-purified ECP revealed no protein content. Additional analysis is necessary to determine the chemical nature of the chemoattractant factors contained in the semi-purified ECP.

As such, differences in chemotactic and chemokinetic activity between the semi-purified FL6-60 parent and RET-04 mutant may be due to varying amounts of chemoattractant constituents contained in the semi-purified fractions. Further chemotactic blind well chamber comparisons of the ECP, standardized by weight, should be conducted to ensure analogous quantities of chemoattractant components are present in both semi-purified ECP. Also, Weeks-Perkins & Ellis (1995) suggested

a major chemotactic component of *Aeromonas salmonicida* was the 50 kDa cell associated proteins comprising the A-layer, and differences were noted in levels of macrophage migration induced by an A-layer positive virulent isolate and an A-layer negative attenuated isolate. In a study conducted by Arias, Shoemaker, Evans & Klesius (2003), compositional differences in LPS expressed by the virulent parent and attenuated RE-33 rifampicin-mutant *E. ictaluri* isolates were analysed. It was reported the RE-33 mutant was deficient in the production of high molecular weight LPS exhibited by the parent (Arias *et al.* 2003). The insignificant amounts of LPS present in the non-purified ECP from the mutant (0.0171 ng mL^{-1}) and from the parent (0.0416 ng mL^{-1}) suggested that LPS was not the chemotaxin. Additionally, the semi-purified ECP had no detectable levels of LPS by the LAL assay.

Overall, both semi-purified ECP induced primarily chemotactic migration as illustrated by the neutralization of migration seen in replicates with equal ECP quantities in both the upper and lower chambers. In the lower surface assay, higher mean macrophage migration was observed for both semi-purified ECP in comparison with their non-purified counterparts at 50% and 70% ECP concentrations in the lower chamber. Additionally, for both ECP fractions the activity of the semi-purified preparations appeared more chemotactic in the checkerboard assay than did the non-purified fractions, especially considering the neutralization of migration displayed in this trial. However, direct comparisons of macrophage behaviour elicited by the non-purified and semi-purified fractions may need further consideration given the greater amount of macrophage movement observed for the control replicates in the semi-purified ECP study. Additional study of the differences in chemoattractant properties between the non-purified and semi-purified ECP is warranted.

Early stages of the inflammatory response are characterized by the migration of leucocytes from the blood and surrounding tissues to the site of injury. The initial activity of phagocytes often determines the outcome of a microbial infection (Griffin 1984; Weeks *et al.* 1988; Weeks-Perkins & Ellis 1995; Klesius & Sealey 1996). The defence against edwardsiellosis in fish is significantly impacted by the activity of macrophages (Miyazaki & Egusa 1976a,b; Miyazaki & Kaige 1985; Padrós, Zarza, Dopazo, Cuadrado & Crespo 2006). Mutoloki, Brudeseth, Reite & Evensen (2006) reported *A. salmonicida*-derived ECP-induced inflammatory reactions and may have enhanced the production of chemotactic signals in Atlantic salmon, *Salmo salar* L. Similarly, the excretion of ECP by *E. tarda* could contribute to the localized effects of this bacterium on specific organs through chemoattractant abilities and proinflammatory action. The results of the present study found *E. tarda* ECP exhibited some chemotactic impact on Nile tilapia macrophages, although most activity appeared to be chemokinetic. Stimulation by *E. tarda* ECP could, to some extent, directly lead to the migration of macrophages toward the site of bacterial invasion, but more importantly could aid in initiating the production and release of host-derived chemoattractant substances, such as components of the complement system, involved in the mediation of the inflammatory response (Nash *et al.* 1986; Klesius & Sealey 1996; Ellis 2001; Klesius *et al.* 2007).

The pathogenesis of *E. tarda* in fish has yet to be fully elucidated. Numerous virulence factors, including extracellular toxin production (Ullah & Arai 1983a,b; Suprapto *et al.* 1995, 1996; Tan *et al.* 2002), have been implicated in edwardsiellosis. As previously mentioned, the stimulation of phagocyte migration by the excreted products of *E. tarda* to the source of microbial invasion is potentially an important defence mechanism of the host against this disease. Nevertheless, the interaction of this bacterium with macrophages could actually play a role in the manifestation of disease. *E. tarda* has been reported to successfully invade host phagocytic cells and avoid killing by phagocyte mediated processes (Ainsworth & Chen 1990; Srinivasa Rao, Lim & Leung 2001; Srinivasa Rao, Yamada & Leung 2003). Potentially, attraction of macrophages by *E. tarda* and its secreted products could function to advance the dissemination of the pathogen and contribute to the development of septicaemia (Klesius & Sealey 1996; Srinivasa Rao *et al.* 2001; Padrós *et al.* 2006). While this investigation indicates ECP of FL6-60 parent and RET-04 mutant *E. tarda* are capable of stimulating Nile tilapia macrophage migration, further study is necessary to determine the specific nature of these host–pathogen interactions and the potential contributions to immune defence and virulence.

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